Atrovenetin as a Potent Antioxidant Compound from *Penicillium* Species

Yukihiro Ishikawa*[a], Kyozo Morimoto[b] and Shigeyasu Iseki[c]
[a]College of Education, Tottori University, Tottori 680, Japan, [b]Hiroshima Prefectural Food Technological Research Center, Hiroshima 732, Japan and [c]Faculty of Agriculture, Tottori University, Tottori 680, Japan

We have established a program to screen large numbers of wild-type fungi isolated from soil for potential producers of antioxidants and synergists for tocopherol (Toc). An antioxidant and potent Toc synergist was isolated from mycelial mats of *Penicillium paraherquei*. It was one of the deoxyherqueinone-type phenalenones and has been identified as atrovenetin.

Under autoxidation conditions, the induction periods of this compound (0.025%) tested by the active oxygen method were 571 and 171 hr in lard with and without Toc (0.04%), respectively. It was also capable of stabilizing vegetable oils such as soybean, rapeseed and palm oils.

KEY WORDS: Antioxidant, atrovenetin, fungal, herqueinone, microorganism, *Penicillium*, synergist, tocopherol.

Several sources of natural antioxidants with characteristic properties are known, including plants and microorganisms (1). In addition to evaluating such antioxidants for use as food additives, many studies have examined them for anticarcinogenesis (2) and for reduction of lipid peroxidation (3). Many antioxidants are being identified as anticarcinogens (4) and are expected to function as internal defense agents (5). Therefore, it appears useful to develop new types of antioxidants and to seek synergists for tocopherol (Toc).

We have given added attention to microbial antioxidants and Toc synergists because microorganisms may produce many new forms. Aoyama *et al.* (6) isolated the antioxidative phenolic compounds—citrinin, protocatechuic acid and curvulic acid—from fungi. Later, Nakakita *et al.* (7,8) identified several antioxidants from a culture filtrate of *Penicillium janthinellum*. We also have reported that some microbial metabolites are potent antioxidants and Toc synergists in several fat substrates with excellent activity compared to that of Toc alone (9–14).

The present paper deals with the isolation and characterization of a new antioxidant and Toc synergist, atrovenetin, from a strain of *Penicillium* species.

EXPERIMENTAL PROCEDURES

Isolation of fungi and cultivation. One gram of soil taken in Tottori, Japan, was suspended in 5 mL of sterilized water. A loop of the suspension was transferred to 5 mL of sterilized water, spread on a medium containing 2.0% malt extract, 2.0% glucose, 0.1% polypepton (from casein), 2.0% agar and 100 ppm streptomycin sulfate, and incubated at 24°C for several days. Colonies grown on the medium were isolated on slants supplemented with the above medium without streptomycin sulfate. Large cultures of the isolated fungi for metabolite preparations were accomplished in surface culture at 24°C for two weeks on a medium composed of 2.0% malt extract, 3.0% glucose and 0.3% polypepton.

Antioxidative activity. The active oxygen method (AOM) was tested by following the Japan Oil Chemists' Society Standard Method, and a simulated AOM was done as follows: Lard (7 g) containing Toc mixture (0.04%) was placed in a test tube (18mm id) and kept at 100°C. Then, air was blown through the oil with a flow rate of 30 mL/min. An induction period was expressed as the number of hours required for the peroxide value to reach 100 meq/kg.

Instrumental analyses. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX-270 NMR spectrometer with tetramethylsilane as an internal standard. Samples were dissolved in deuterated chloroform or dimethyl sulfoxide. Infrared (IR) spectra were analyzed with a Hitachi Model 260-30 infrared spectrophotometer (Hitachi Co., Tokyo, Japan) on samples prepared as potassium bromide disks. Mass spectra were recorded on a Hitachi M80B mass spectrometer in the electron impact mode.

RESULTS AND DISCUSSION

Screening of fungi. We first screened for fungal isolates to find those that produce Toc synergists. Acetone extracts of mycelial mats and ethyl acetate extracts of culture broth were used to estimate each isolates Toc synergism in lard *via* the simulated AOM. One of the most active strains (No. 40-3) was isolated from soil. From its acetone extract, an Emmerie-Engel's (EE) reagent-positive (greenish brown) and UV-absorptive substance was observed at Rf = 0.2 on a thin-layer chromatography (TLC) plate [pre-coated Kieselgel 60 F_{254}, Merck, Darmstadt, Germany; ethyl acetate/acetone (6:4) as a mobile phase].

Strain identification. For the morphological investigation of strain 40-3, it was grown on malt extract agar, potato dextrose agar (Difco Laboratories, Detroit, MI) and Czapek agar plates at 24°C for a week and then left for about 10 days at room temperature.

Microscopic observation of the strain showed characteristic features of *Penicillium* species (Fig. 1), and its diagnosis is as follows: Colonies on malt extract agar, potato dextrose agar, and Czapek agar after one week are attaining diameters of 55–70 mm, 40–60 mm, and 15 mm, respectively; typically velutinous with a dark yellow green; penicilli biverticillate symmetrical; conidiophores typically granular walled; conidia predominantly elliptical with roughness arranged in a spiral pattern. These results indicate that the strain is *Penicillium paraherquei* (15,16).

Isolation of metabolite and its identification. Mycelial mats (6-L cultures) were dried *in vacuo* and extracted with acetone several times. These extracts were combined and concentrated to give a precipitate. After drying, the precipitate was dissolved in a small amount of acetone and

*To whom correspondence should be addressed.

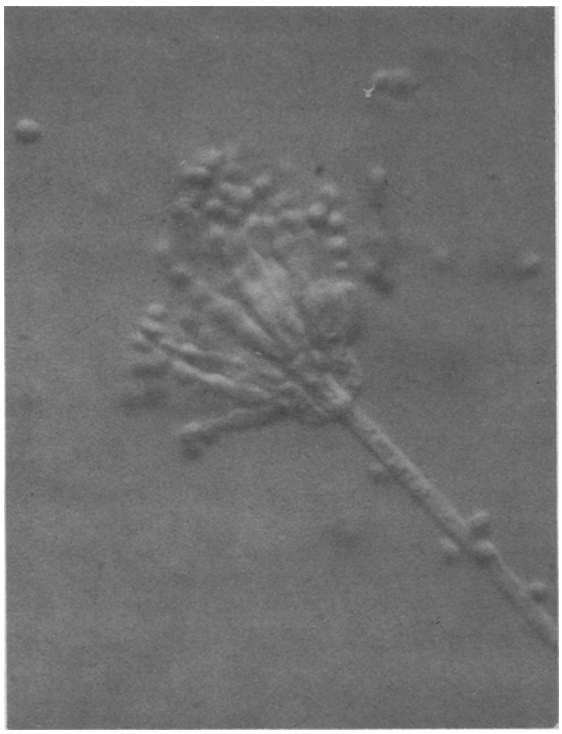

FIG. 1. Photomicrograph of strain 40-3.

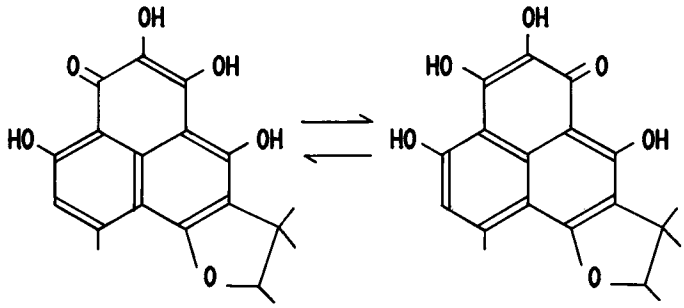

FIG. 2. The structure of enantiomers of atrovenetin.

filtered. The filtrate was discarded. The residue on a filter paper was dissolved in methanol at 60°C and insoluble matters were filtered off. Methanol-soluble matters were concentrated to give *ca.* 1 g of a brownish yellow substance, which we designated 40-3M. 40-3M Was recrystallized as fine needles from methanol.

The analytical data of 40-3M are as follows: Analysis (Calcd. for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30%; Found: C, 66.80; H 5.63%). MS (20eV): *m/z* (rel int %) 342(M^+, 100), 327(100), 313(17), 299(14), 297(15). ^{1}H-NMR(DMSO-d_6): δ 1.26(3H, s), 1.44(3H, d, J=6.7Hz), 1.51(3H, s), 2.75(3H, s), 4.68(1H, q, J=6.7Hz), 6.84(1H, s). ^{13}C-NMR (DMSO-d_6): δ 14.6, 20.8, 23.3, 25.6, 43.0, 90.9, 104.3, 104.8, 107.5, 116.0, 118.3, 124.1, 131.7, 144.0, 162.1, 165.0, 169.3. IR (KBr, cm^{-1}): 3300(br), 1605, 1270, 1170, 1140. UV(EtOH, λ): 217(17160), 260(sh, 7580), 282(sh, 3510), 382(7140).

The ^{1}H-NMR spectrum of 40-3M showed that the number of protons is fourteen, and short four in comparison with the number in the molecular formula. The facts that 40-3M gave a greenish brown color with EE reagent and has antioxidant activity suggest the presence of hydrogenated and free hydroxy groups in the molecule. On methylation with methyl iodide and potassium carbonate in acetone, 40-3M gave a major yellow compound, which provided a single spot on TLC (Rf=0.65). The ^{1}H-NMR spectrum ($CDCl_3$) of the methyl ester showed two signals at δ 4.018(6H,s) and 4.021(3H,s) corresponding to methoxyl groups, and one signal at δ 18.12(1H,s) corresponding to a hydrogenated hydroxy group. On acetylation with acetic anhydride and pyridine, 40-3M gave two yellow compounds on TLC (Rf=0.2 and Rf=0.03). The compound at Rf = 0.03 was repeatedly purified by high performance liquid chromatography (HPLC) (Column, 4-250mm, LiChrosorb SI 60, 5 micron particle size, Merck; mobile phase, chloroform). The ^{1}H-NMR spectrum ($CDCl_3$) of the compound provided further proof by showing signals at δ 2.35, 2.37, 2.38 and 2.52 for acetyl groups, indicating that three hydroxy groups, as well as one hydrogenated hydroxy group, are present in 40-3M. Absorption bands at 1140 and 1170 cm^{-1} in the IR spectrum are characteristic of a cyclic ether. The ^{13}C-NMR spectrum of 40-3M showed a shortage of two carbons due to overlapping, unresolved singlets centered at δ 165.0 and 169.3.

The chemical shifts of the carbons are similar to those of deoxyherqueinone from *P. herquei* (17), which produces a group of closely related compounds with a phenalenone skeleton and a 1,2,2-trimethyltetrahydrofuran ring (18,19). The molecular weight of 40-3M indicates that it must be a demethylated form of deoxyherqueinone, atrovenetin (20) or demethylherqueichrysin (21). On acetylation with acetic anhydride and pyridine, demethylherqueichrysin gave its tetra-acetate and signals of a six-proton singlet due to acetyl groups appearing at δ 2.32 and 2.35 (21). Demethylherqueichrysin has no chelated hydroxy proton in its molecule.

Ayer *et al.* (20) reported that atrovenetin was reacted with diazomethane in dichloromethane to afford two kinds of atrovenetin trimethyl ether. The ^{1}H-NMR data of one of them agreed closely with those of methylated 40-3M. However, our data were quite different from those for the compound synthesized by Buchi and Leung (22). This difference is attributed to the presence of enantiomers of atrovenetin (20). Authentic atrovenetin obtained from *P. herquei* (23) gave ^{1}H- and ^{13}C-NMR spectra identical with those of 40-3M. Therefore, 40-3M has been identified as atrovenetin. The structure of 40-3M is shown in Figure 2.

Atrovenetin was isolated from *P. atrovenetum* (24), *P. herquei* (23) and *Gremmeniella abietina* (20). It has been found to show antibiotic activities on *Bacillus subtilis* and *Staphylococcus aureus*. In a mutation assay with *Salmonella typhimurium*, 40-3M concentrations greater than 100 μg/plate were weakly inhibitory to the growth of the strain (unpublished data).

Antioxidative activity. The antioxidant activity of 40-3M and its synergism with Tbc were tested under AOM conditions. Figure 3 demonstrates that 40-3M is a potent antioxidant and excellent Tbc synergist in lard. As shown in Table 1, 40-3M at a concentration of 0.05% markedly stabilized vegetable oils tested, especially rapeseed oil.

A new finding that atrovenetin acts as a potent antiox-

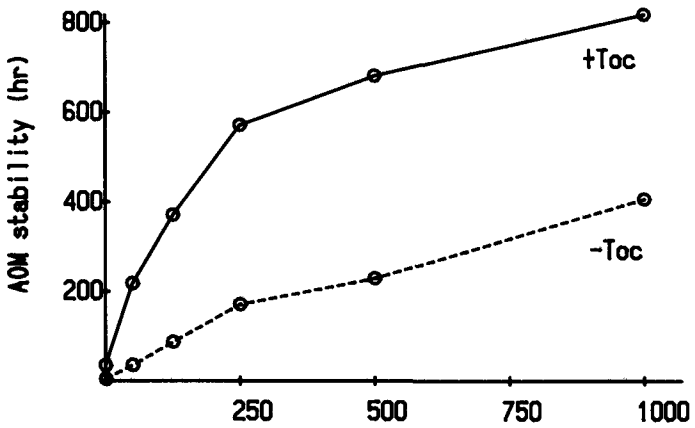

FIG. 3. Antioxidative activity of atrovenetin and its synergism with tocopherol (0.04%) in lard.

TABLE 1

Effect of Atrovenetin on the Stability of Vegetable Oils

Oil	AOM stability (hr)	
	None	+ 40-3M[a]
Soybean	12.9	241
Rapeseed	13.8	370
Corn	16.5	250

[a]Concentration is 0.05%.

idant was revealed as a result of these investigations. As reported in previous papers (12,14), new herqueinone-related antioxidants have been isolated from *Penicillium* species and exhibited excellent synergistic activities with Toc. The correlation between structure and antioxidation of herqueinone derivatives is now being investigated.

ACKNOWLEDGMENT

The authors are grateful to T. Hirata for NMR data of herqueinone derivatives. We thank G. Buchi and L.C. Vining for a sample of atrovenetin. T. Ito provided valuable advice as to the identification of *Penicillium* species. The study received financial support in part from the Itoh Memorial Foundation, Japan.

REFERENCES

1. Dugan, L.R., *Autoxidation in Food and Biological Systems*, Plenum Press, New York, 1980, pp. 261-282.
2. Floyd, R.A., *Free Radicals and Cancer*, Marcel Dekker, Inc., New York, 1982, pp. 201-244 and 361-396.
3. Ogura, R., *Lipid Peroxides in Biology and Medicine*, Academic Press, Orlando, 1982, pp. 255-269.
4. Ames, B.N., *Science 221*:1256 (1983).
5. Fukuzawa, K., *Free Radicals in Clinical Medicine, Vol. 3*, Nihon-Igakukan, Tokyo, 1988, pp. 41-51.
6. Aoyama, T., Y. Nakatani, M. Nakagawa and H. Sakai, *Agric. Biol. Chem. 46*:2369 (1982).
7. Nakakita, Y., K. Yomosa, A. Hirota and H. Sakai, *Ibid. 48*:239 (1984).
8. Nakakita, Y., T. Aoyama and H. Sakai, *Ibid. 48*:2385 (1984).
9. Ishikawa, Y., K. Morimoto and T. Hamasaki, *J. Am. Oil Chem. Soc. 61*:1864 (1984).
10. Ishikawa, Y., K. Morimoto and T. Hamasaki, *J. Food Sci. 50*:1742 (1985).
11. Ishikawa, Y., K. Morimoto and T. Hamasaki, *J. Jpn. Oil Chem. Soc. 35*:903 (1986).
12. Morimoto, K., T. Yoshiwa, Y. Ishikawa and T. Hamasaki, *Ibid. 36*:10 (1987).
13. Ishikawa, Y., K. Morimoto and T. Hamasaki, *Ibid. 37*:51 (1988).
14. Ishikawa, Y., and S. Iseki, *Proc. ISF-JOCS World Congress 1988*, 1989, p. 324.
15. Raper, K.B., and C. Thom, *A Manual of the Penicillia*, The Williams and Wilkins Co., Baltimore, 1949, pp. 119-125.
16. Ramirez, C., *Manual Atlas of the Penicillia*, Elsevier Biomedical Press, Amsterdam, 1982, pp. 518-521.
17. Suga, T., T. Yoshioka, T. Hirata and T. Aoki, *Bull. Chem. Soc. Jpn. 56*:3661 (1983).
18. Simpson, T.J., *J. C. S. Chem. Comm.*, 258 (1976).
19. Simpson, T.J., *J. C. S. Perkin I*, 1233 (1979).
20. Ayer, W.A., M. Soledade Pedras and D.E. Ward, *Can. J. Chem. 65*:760 (1987).
21. Frost, D.A., D.D. Halton and G.A. Morrison, *J. C. S. Perkin I*, 2443 (1977).
22. Buchi, G., and J.C. Leung, *J. Org. Chem. 51*:4813 (1986).
23. Narasimhachari, N., K.S. Gopalkrishnan, R.H. Haskins and L.C. Vining, *Can. J. Microbiol. 9*:134 (1963).
24. Neill, K.G., and H. Raistrick, *Biochem. J. 65*:166 (1957).

[Received September 26, 1990; accepted June 24, 1991]